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Functional Characterization of the P2 A Initiator Protein and Its DNA Cleavage Site

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The A protein of bacteriophage P2 initiates DNA replication by a single-stranded cut at the origin, and the DNA replication proceeds unidirectionally by a modified rolling circle type of replication. The P2 A protein belongs to a family of proteins involved in the initiation of rolling circle DNA replication, and the prototype for this family is the well-characterized A protein of phage ϕX174. One of the common motifs of this family contains two conserved tyrosine residues, which have been shown to be able to alternate in catalyzing the cleavage as well as joining reactions in the ϕX174 A protein. We investigated the role of the conserved tyrosine residues in P2 A protein by *in vitro* mutagenesis. Only one of the two conserved tyrosine residues was found to be involved in the cleavage reaction. The tyrosine residue dispensable for cleavage and ligation is, however, required at some other stage of the P2 growth cycle, since viable recombinants containing this mutation could not be obtained. The sequence requirements for cleavage of the target site were analyzed with a set of oligonucleotides having single base alterations in the nick region, and the results indicate that only five core nucleotides need to be conserved for efficient cleavage.

INTRODUCTION

Bacteriophage P2 DNA replication is initiated by a single-stranded, site-specific cleavage at the origin of replication (*ori*), and it proceeds unidirectionally by a modified rolling circle type of replication (Schnös and Inman, 1971; Geisselsoder, 1976; Chattoraj, 1978). The cleavage reaction is mediated by the *cis*-acting P2 A protein, which becomes covalently attached to the 5′ end of the cleaved DNA (Lindahl, 1970; Liu and Haggård-Ljungquist, 1994). The *ori* has been located within the coding part of the *A* gene, in a region devoid of AT rich sequences and inverted or direct repeats (Schnös and Inman, 1971; Liu *et al.,* 1993), commonly associated with prokaryotic origins of replication (Bramhill and Kornberg, 1988).

The *A* gene codes for a protein of 86.3 kDa, and the purified A protein cleaves single-stranded, but not linear double-stranded, *ori* containing DNA (Liu and Haggård-Ljungquist, 1994; Liu *et al.,* 1993). By comparative analysis, the proteins involved in the initiation of rolling circle DNA replication have been divided in two classes, the replication (Rep) and the mobilization of plasmids (Mob) class, with a common motif [HisUHisUUU (motif 2)] believed to be involved in metal ion coordination (Ilyina and Koonin, 1992; Koonin and Ilyina, 1993). The Rep class has further been subgrouped into two large superfamilies and several smaller families, where the members of each family show statistically significant similarities (Koonin and Ilyina, 1993). The P2 A protein belongs to superfamily I, which has two additional conserved motifs in common. Motif 1 is located upstream of motif 2, and motif 3 downstream. The C-terminal motif 3 contains the two tyrosine residues believed to form the covalent link with the nicked DNA. The prototype of this family is the A protein of ϕX174, where the two tyrosine residues are able to alternate in catalyzing the cleavage and joining reactions (Van Mansfeld *et al.,* 1986; Hanai and Wang, 1993). To test whether the corresponding two conserved tyrosine residues in the P2 A protein have a similar role, we mutated them *in vitro* and studied their capacity to cleave single-stranded DNA. We find, however, that one of the tyrosine residues of P2 A protein is dispensable in the cleavage reaction *in vitro,* although both are required for normal *in vivo* phage growth.

MATERIALS AND METHODS

Biological materials

For bacterial strains, phage, and plasmids, see Table 1.

Plasmid constructions

Pertinant regions of the *A* gene and the oligonucleotide primers used to construct the plasmids overexpressing the mutated P2 *A* genes are shown in Fig. 1. Standard molecular cloning techniques were performed as described in Sambrook *et al.* (1989). Unless otherwise stated, the enzymes were purchased from Pharmacia and used as recommended by the manufacturer.

Construction of plasmid pEE712 which overexpresses the mutated A protein, Y450D. The mutated A gene was constructed by two consecutive PCR runs. In the first run two fragments were amplified using primers 83.3R and

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TABLE 1

Bacterial Strains, Phage, and Plasmids

Strain, phage, or plasmid	Pertinent features	Origin or reference
Bacterial strains		
C-1a	Prototrophic *E. coli* C strain	Sasaki and Bertani, 1965
C-2073	C-1a lysogenized by P2 *c*5 Aam127	Nilsson and Bertani, 1977
BL21 (DE3)	*E. coli* B strain carrying the T7 RNA polymerase gene under the control of the *lac* promoter	Studier *et al.,* 1990
Phage		
P2 *Aam127*	P2 mutant containing an amber mutation in the *A* gene	Lindahl, 1971
Plasmids		
pET16b	A pET expression plasmid containing a His-Tag under the control of the T7 promoter ϕ10	Studier *et al.,* 1990 Novagen, Madison, WI
pEE711	pET16b expressing the wild-type *A* gene fused to the His-Tag of the vector	Liu and Haggård-Ljungquist, 1994
pEE712	As pEE711 but the A gene contains the Y450D mutant	This paper
pEE713	As pEE711 but the A protein contains the Y454F mutation	This paper
pEE714	As pEE711 but the A gene contains the Y450D and the Y454F mutations	This paper
pEE715	As pEE711 but the A protein lacks the 302 first amino acids	This paper
pEE717	pET16b derivative containing the 87.4 to 90.9% region of P2 DNA containing the Y454F mutation	This paper

88.0L-mutY450D, and 88.0R-mutY450D and 90.0L, respectively (Fig. 1). In the next PCR run the two amplified fragments were used as substrate for the amplification using primers 83.3R and 90.0L. The generated 83.3 to 90.0% fragment, containing the Y450D mutation, was phosphorylated using T4 polynucleotide kinase, and ligated with vector pET16b, which had been cleaved with *Nde*I and the generated cohesive ends were filled in using the Klenow enzyme and the 5′ phosphates removed using alkaline phosphatase. After transformation into strain C-1a, the DNA sequence of the whole cloned gene and the junction to the His-Tag of the vector was confirmed by DNA sequence analysis.

Construction of plasmid pEE713, which overexpresses the mutated A protein, Y454F. The plasmid was constructed in two steps. First the C-terminal part of the A gene, the 87.4 to 90.9% region containing the Y454F mutation, was amplified using primers 87.4R-mutY454F and 90.9L (Fig. 1). The amplified fragment was phosphorylated using T4 polynucleotide kinase and ligated with vector pET16b, which had been cleaved with *Nde*I as before, generating plasmid pEE717. That the plasmid had the cloned fragment integrated in the correct orientation, and that the PCR reaction had not induced any mutations, was verified by DNA sequence analysis. Since the vector contains only one *Nru*I site, and the *A* gene only contains one *Nru*I site, located between tyrosines 450 and 454 (Fig. 1), the N-terminal part of the *A* gene could be inserted into plasmid pEE717 by exchanging the *Nru*I fragment of plasmid pEE717 with that of plasmid pEE711, which contains the wild-type A gene fused to the His-Tag of the vector, generating plasmid pEE713.

Construction of plasmid pEE714 which expresses the double-mutated A protein, Y450D + Y454F. The N-terminal part of the A gene, containing the Y450D mutation contained on the *Nru*I fragment of plasmid pEE712 was exchanged with the *Nru*I fragment of plasmid of pEE717 as described above, thus generating the *A* gene with both the Y450D and Y454F mutations fused in frame with the His-Tag of the vector.

Construction of plasmid pEE715 which overexpresses the truncated A protein, A_{303}. An in-frame fusion between the His-Tag of pEE716 and the internal start site at Met-303 was made by amplification of the

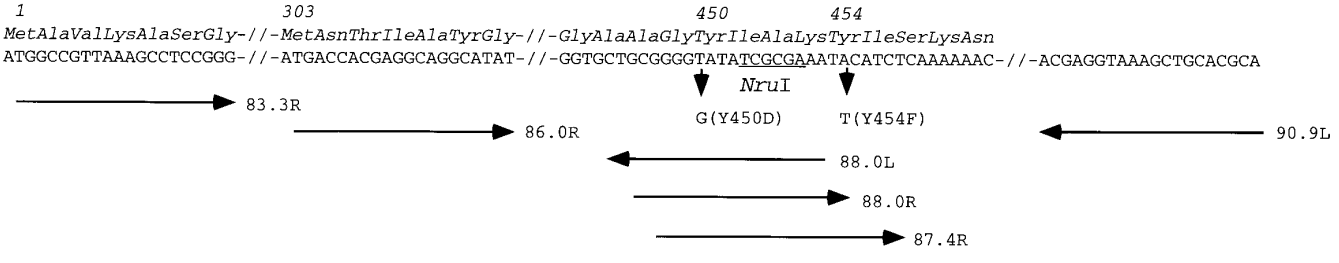

FIG. 1. DNA sequence of pertinent parts of the *A* gene and the derived amino acid sequence. The numbers above the sequences refer to the amino acids of the A protein. The horizontal arrows indicate the orientation and location of the respective primer used for plasmid constructions. The vertical arrows indicate base changes introduced by PCR mutagenesis (See Materials and Methods).

pertinent region using primers 86.0R and 90.9L (Fig. 1). After phosphorylation, the amplified fragment was inserted into the vector pET16b, which had been cleaved with *Nde*I as before. After transformation into strain C-1a, the DNA sequence of the whole cloned gene, and the junction to the His-Tag of the vector was confirmed by DNA sequence analysis.

Protein purification

Each plasmid was transformed into strain BL21(DE3) for production of the proteins. Overexpression of gene A leads to formation of inclusion bodies. The A protein was purified to about 90% homogeniety by one-step affinity chromatography under denaturing conditions, followed by renaturation as described previously (Liu and Haggård-Ljungquist, 1994). Protein concentrations were determined by the method of Bradford, using bovine serum albumin as a standard (Bradford, 1976), and adjusted to approximately 150 ng/μl.

Amplification of DNA by the polymerase chain reaction (PCR)

The amplification was performed as described before (Liu and Haggård-Ljungquist, 1994).

DNA sequencing

The sequence analyses were performed by the chain terminating technique (Sanger *et al.,* 1977).

In vitro cleavage and joining reactions

The synthetic oligonucleotides (150 pmol) used as substrates were 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (Amersham), and unincorporated nucleotides were removed. The cleavage and joining reactions (20 μl) contained 6 pmol of the labeled oligonucleotide and 450–750 ng of the purified wild-type or mutated A protein under the same conditions as described before (Liu and Haggård-Ljungquist, 1994). The products were separated on a 20% polyacrylamide gel containing 8 *M* urea, followed by autoradiography of the dried gels. The reaction products were quantified using storage phosphor technology.

RESULTS

Kinetics of the P2 A protein cleavage reaction

The P2 A protein has been shown to efficiently cleave single-stranded P2 *ori*-containing DNA (Liu and Haggård-Ljungquist, 1994). The initiation proteins of rolling circle replication are known to catalyze a cleavage as well as a joining reaction, which can be expected to be in equilibrium with each other. The kinetics of the cleavage reaction were studied using increasing amounts of purified A protein and a constant amount of a 5′-labeled oligonucleotide 20 nucleotides (nt) long containing the *ori* sequence. Since the A protein remains covalently joined to the 5′ end after cleavage, the reaction should generate a free 5′ end-labeled fragment 8 nt long. As can be seen in Fig. 2, the cleavage reaction yields a labeled fragment of 8 nt and the reaction reaches almost 100%. This implies that under the conditions used, the joining reaction is either less efficient than the cleavage reaction or does not occur at all. At low concentrations of protein A the curve shows evidence of cooperativity, which may indicate that the active form of the A protein is at least a dimer.

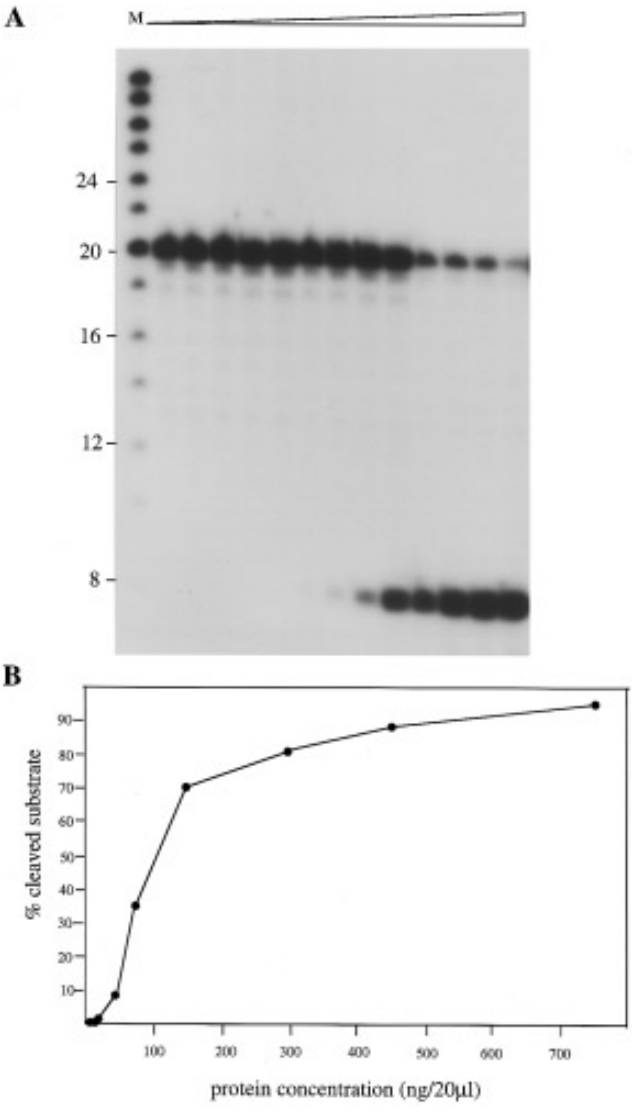

FIG. 2. Site-specific cleavage of a single-stranded *ori* containing oligonucleotide by the A protein. Varying amounts of the A protein were incubated with 5′ end-labeled oligonucleotide ori (5′-GCGCCTCG↓G-AGTCCTGTCAA) as described under Materials and Methods. (A) Autoradiograph of the polyacrylamide gel. Lane 1, 5′ end-labeled size markers ranging from 8 to 32 nt in length (Pharmacia). Lane 2 to 14 contains increasing amounts of the A protein. (B) The percentage of cleaved oligonucleotide was determined from the gel shown above by storage phosphor technology.

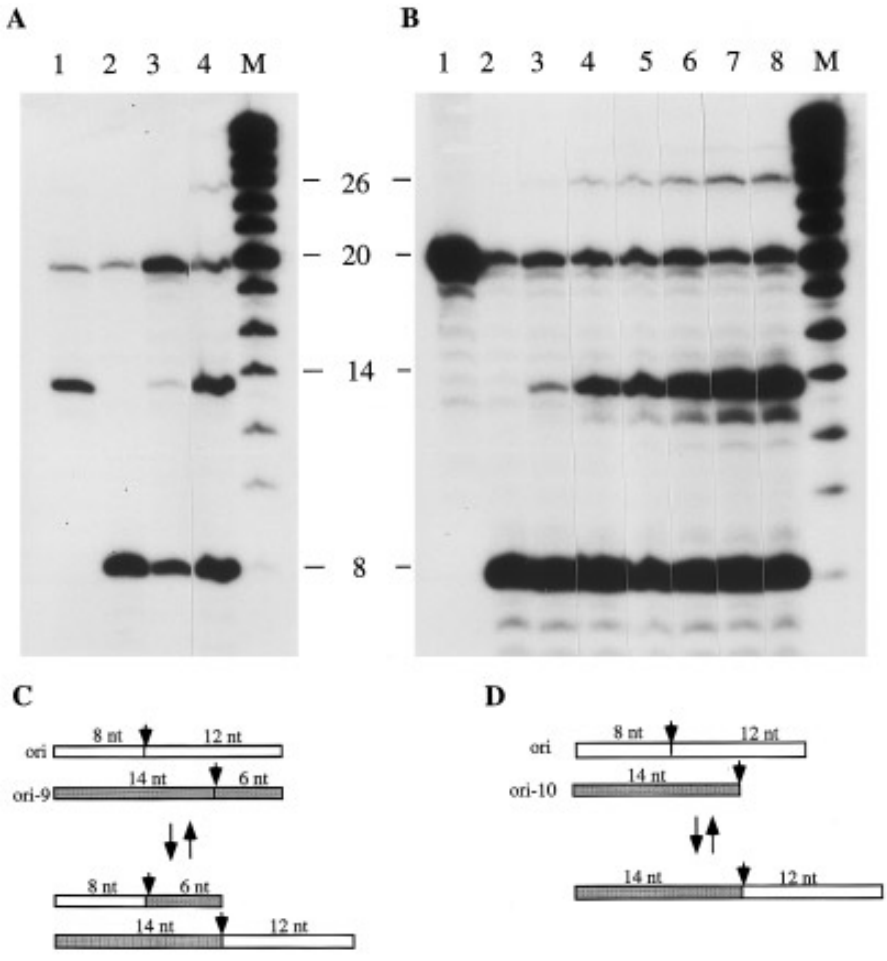

FIG. 3. Site-specific cleavage and joining reactions. (A) The A protein was incubated with oligonucleotides ori (5′-GCGCCTCG↓GAGTCCTGTCAA) and/or ori-9 (5′-ATCGCCGCGCCTCG↓GAGTCC). Lane 1, only 5′ end-labeled ori-9; lane 2, only 5′ end-labeled ori; lane 3, 5′ end-labeled ori and unlabeled ori-9; lane 4, 5′ end-labeled ori and ori-9; lane M, size markers (Fig. 2). (B) The A protein was incubated with a constant amount of 5′ end-labeled oligonucleotide ori and varying amounts of 5′ end-labeled ori-10 (5′-ATCGCCGCGCCTCG↓) as described under Materials and Methods. Lane 1, 5′ end-labeled ori in absence of A (control); lane 2, 5′ end-labeled ori; lanes 3–8, constant amount of 5′ end-labeled ori and increasing amounts of ori-10 (6 to 55 pmol per reaction) lane M, size markers. Schematic drawings of the respective reaction are indicated in C and D.

The P2 A protein catalyzes joining of single-stranded oligonucleotides

The P2 A protein is expected to cleave as well as join the DNA strands after one round of replication, giving rise to monomeric circles in a mode similar to the ϕX174 RF replication. Under the conditions described above we found no indication of a joining reaction. In order to detect a possible joining reaction, two oligonucleotides which differed in the location of the nick site, 8 (ori) and 14 nt (ori-9) from the respective 5′ end (Fig. 3C), were incubated with the P2 A protein in the same reaction mixture. Either one or both of the oligonucleotides were labeled at the 5′ end in order to define the origins of the hybrid products. The presence of the labeled hybrid length product would indicate ligation activity of the P2 A protein. As can be seen in Fig. 3A, the labeled 8-nt cleavage product is able to join the unlabeled 6-nt cleavage product generating a new 14-nt-long labeled fragment (lane 3), and the labeled 14-nt cleavage product is able to join the unlabeled 12-nt cleavage product generating a new 26-nt-long fragment (lane 4).

In the experiments described above, the fragments joined were the products of a preceding cleavage reaction. To directly study only the joining reaction, a constant amount of 5′ labeled oligonucleotide ori and an increasing amount of 5′ labeled oligonucleotide ori-10 (which is a 14-mer ending at the 3′ end of the *ori* cleavage site) were incubated with the P2 A protein. The joining of the labeled 14-nt ori-10 to the unlabeled 12-nt cleavage product generates a 26-nt-long fragment as expected (Figs. 3B and D), but the joining reaction is rather inefficient compared to the cleavage reaction. It seems as if the balance between the cleavage and the joining reaction in our system is shifted toward cleavage, since the amount of joined product never exceeds 4%.

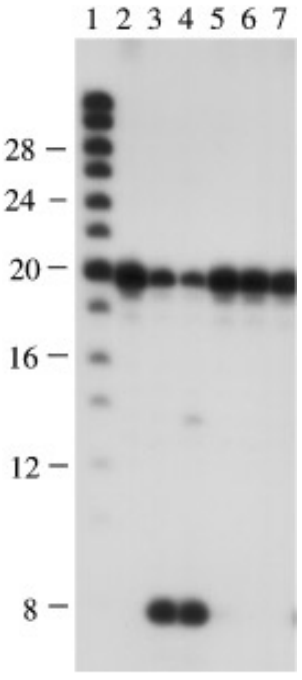

FIG. 4. Site-specific cleavage reactions with wild-type, mutated, and truncated A proteins. The A proteins were incubated with 5′ end-labeled oligonucleotide ori (Fig. 2) as described under Material and Methods. The products were separated by polyacrylamide gel electrophoresis and autoradiographed. Lane 1, size markers (Fig. 2); lane 2, no A protein; lane 3, wild-type A protein; lane 4, Y450D-mutated A protein; lane 5, Y454F-mutated A protein; lane 6, Y450D + Y454F-mutated A protein; lane 7, truncated A_{303} protein.

Tyr-454, but not Tyr-450, is required for the cleavage reaction

Purified ϕX174 A* protein has been shown to cleave single-stranded oligonucleotides containing the *ori* region (Van Mansfeld *et al.,* 1984; 1986), and mutations in either Tyr-343 or Tyr-347 will not affect the cleavage reaction (Hanai and Wang, 1993). To test if the cleavage reaction of the P2 A protein acts in a similar manner, Tyr-450 was changed to Asp (Y450D) and Tyr-454 to Phe (Y454F) by *in vitro* mutagenesis. After purification, their cleavage capacities were analyzed using the wild-type *ori*-containing oligonucleotide described above. The purified Y450D protein cleaves the 20-nt-long 5′ end-labeled ori oligonucleotide, generating a 8-nt-long labeled fragment like the wild-type A protein (Fig. 4, lanes 3 and 4). However, in contrast to the wild-type A protein, Y450D generated an additional weak fragment of 14 nt. Thus it seems that the Y450D mutant cleaves the *ori*-containing oligonucleotide both at *ori* and 5 nucleotides to the right of *ori,* generating the 14-nt-long labeled fragment. Often a weak 26-nt-long fragment can also be seen which might be the product of a ligation reaction between the 14-nt labeled fragment and the 12-nt unlabeled fragment generated by cleavage at *ori.* The Y454F protein, and the protein containing both mutations (Y450D + Y454F) were unable to cleave the oligonucleotide (Fig. 4, lanes 5 and 6).

The A gene contains several possible internal translational initiation sites (Liu *et al.,* 1993). A truncated form of the A protein has been purified, which starts at amino acid 303 which is 19 amino acids upstream of motif 1, e.i. at a location reminiscent of the ϕX174 A* protein which is initiated 27 amino acids upstream of motif 1. The truncated P2 A protein, A_{303}, was also tested for the capacity to cleave the *ori*-containing oligonucleotide, but the truncated A protein was found to have no cleavage activity (Fig. 4, lane 7).

Effects of the Y450D mutation on phage growth

Since the Y450D-mutated A protein is able to cleave as well as join single-stranded *ori*-containing oligonucleotides like the wild-type A protein, we were interested to see if it had any effects on phage growth. To introduce the Y450D mutation into P2, a non-permissive P2 *A*am127 lysogenic strain (C-2073) was transformed with plasmid pEE712, which contains the Y450D-mutated *A* gene or pEE711, which contains the wild-type *A* gene, and free phages able to grow on the nonpermissive strain C-1a were isolated. Plaque formers on the nonpermissive strain could result either from reversion of the *A*am127 mutation or recombination between the plasmid and the prophage. The structure of the homologous region in the phage and plasmid is as follows 615 nt-*A*am127-732 nt-Y450D-970 nt. Thus the double crossover reaction leading to an exchange of the *A*am127 mutation to the wild-type sequence can be expected to contain the Y450D mutation with a high frequency, unless it affects the growth of the phage. Since the Y450D mutation generates an *Eco*RV restriction site (Fig. 1), the pertinent DNA region was amplified by PCR and analyzed for the presence of the *Eco*RV site. Of 30 plaques tested from the P2 *A*am127 lysogen carrying plasmid pEE712, two were found to contain a mixture of the wild-type and the Y450D mutation. However, upon plaque purification of the original plaques containing the mixed bursts, the Y450D mutation is lost (200 single plaques were tested). The control strain containing plasmid pEE711 gave no mixed bursts. Therefore we conclude that the Y450D mutation, even though it cleaves and joins single-stranded *ori*-containing DNA normally, is inhibited at some other stage in the life cycle.

Characterization of the sequence requirements at the cleavage site

To characterize the sequence requirements for the cleavage reaction we used a set of synthetic oligonucleotides containing single base transitions in the nick region. As can be seen in Fig. 5, the two nucleotides upstream of the cleavage site (−1 and −3) are critical for the cleavage reaction, and nucleotides −2, +1, and +2 show a reduced cleavage efficiency. Thus it seems as if

Oligonucleotide	Sequence (5′ to 3′)	Cleavage efficiences
	▼	
ori (wt)	GCGCCTCG GAGTCCTGTCAA	0.8
ori-7	....T..	0.8
ori-8	.....C..	0
ori-1	......T.	0.5
ori-2	.......A	0
ori-3	.... A.....	0.5
ori-4	.... .G.....	0.4
ori-5	....A.....	0.9
ori-6	....C.....	0.7

FIG. 5. Relative cleavage efficiencies of synthetic oligonucleotides containing point mutations in the *ori* region. The location of the nick is indicated by the arrow. The cleavage efficiencies were determined by quantitation of the fraction of cleaved material out of total activity in a phosphoimager. The values were obtained using 150 ng of A protein per reaction.

the core region required for cleavage is 5 nucleotides long.

DISCUSSION

Rolling circle replication is commonly used among circular replicons of different origins, for example single-stranded (ss) and double-stranded (ds) DNA phages (Van Mansfeld *et al.,* 1984; Baas and Jansz, 1988), ss DNA plasmids (Gruss and Ehrlich, 1989; Novick, 1989), ss DNA plant viruses (Stenger *et al.,* 1991; Saunders *et al.,* 1991), and ss and ds DNA animal viruses (Berns, 1990; Dasgupta *et al.,* 1992). In the systems studied, the initiation proteins possess a nicking-closing and topoisomerase-like activity. The best studied system is that of the ss DNA phage ϕX174, where the A protein nicks the *ori* site in the viral strand of the replicative form and forms a covalent link to the 5′ end of the cleaved strand. The 3′ end is thereafter extended by the host polymerase displacing the 5′ viral strand and after one round of replication the parental viral strand is religated and the A protein is transferred to the progeny strand to initiate a new round of replication (Baas and Jansz, 1988). P2 is believed to follow a similar mode of replication, but the details of the P2 replication system have not yet been elucidated. In this work we addressed the following questions about the P2 A initiator protein: (i) can it promote the cleavage as well as the joining reaction, (ii) are the two conserved tyrosine residues involved in the cleavage reaction, and (iii) what are the sequence requirements for the cleavage reaction?

As shown above, the P2 A protein mediates a strand-specific cleavage at *ori,* and it also exhibits a weak ligation activity under the conditions used. Since the purified A protein does not bind to double-stranded DNA either in linear or supercoiled circular form *in vitro* (Liu and Haggård-Ljungquist, 1994), we used single-stranded oligonucleotides as substrates for the cleavage and ligation reactions. The inefficient ligation reaction *in vitro* with ss oligonucleotides is understandable when compared to *in vivo* conditions where the ends are held together after one round of replication by the replication machinery. It is also possible that the optimal conditions for cleavage and ligation are not identical, something that needs to be studied further.

A comparative analysis of the initiator proteins for rolling circle replication places P2 A among the proteins of superfamily I of the Rep class which contain two tyrosine residues in the presumed catalytic site (Koonin and Ilyina, 1993). The prototype of this family is the A protein of ϕX174, which can utilize either of the two tyrosine residues for the cleavage-ligation reaction (Van Mansfeld *et al.,* 1986). Our mutation studies show that Tyr-454 but not Tyr-450 of the P2 A protein is required for the cleavage reaction. However, since the Tyr-450 residue could not be replaced with Asp by recombination into a viable phage, the Tyr-450 residue is most likely critical for P2 A function *in vivo.*

Alignments of the replication *ori* sites of superfamily I of the Rep class has revealed very little sequence conservation, but in common with superfamily II all the known or presumed cleavage sites occur on the 3′ side of a G residue, and in all cases except for P2 and the P2 related phage 186, the 5′ site is an A residue (Koonin and Ilyina, 1993). Also a CT pair seems to be conserved on the 3′ side of the nick in several members of superfamily I. We tested the importance of the conserved residues for the *in vitro* cleavage reaction by introducing single base transitions in the DNA substrate. We found that the T residue of the conserved CT pair as well as the G residue on the 3′ side of the nick were essential for the cleavage reaction. However, making the P2 *ori* sequence more similar to the other members of the family by changing the 5′ G residue to an A led to a decreased cleavage efficiency.

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